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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

This proposal utilizes a novel screening strategy that can be applied to the identification of genes involved in metastasis and invasion. The basic approach is to make retroviral-based expression libraries from invasive tumor tissues, and then transfer the invasive phenotype to non-invasive breast tumor cells. This report describes a screen of a library derived from the highly invasive MBA-MB-231 breast tumor cell line. Four cDNAs were recovered in this screen that exhibited an invasive phenotype when retested in the non-invasive MCF-7 breast cell line. These cDNAs were sequenced in their entirety and were found to encode DAP-1 (a known tumor suppressor), LIPE (hormone sensitive lipase), HSPA5 (heat shock protein) and ABLIM (actin binding protein). The DAP-1 cDNA was orientated in the anti-sense, and was by far the most invasive (20-fold increase vs vector). Since DAP-1 had already been attributed tumor suppressor properties in other biological systems, it was selected for further analysis. Anti-sense expression of DAP-1 in NIH 3T3 mouse fibroblasts caused transformation, which was associated with specific activation of the small GTPase RhoA. To summarize, we have successfully screened for invasive cDNAs, and are now characterizing these cDNAs to determine the molecular basis of their transforming activity.

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INTRODUCTION:

We have demonstrated previously that retroviral-based expression screening is a powerful approach for the identification of cDNAs whose overexpression are associated with growth deregulation (1). In this proposal, an expression system that was developed by the principle investigator has been adapted for the identification of genes that contribute to the progression of breast cancer cells to a metastatic state. Here we report the successful isolation of four cDNAs from an MBA-MB-231 derived library that can confer a phenotype of increased invasiveness when overexpressed in MCF-7 cells. One of these cDNAs contains the coding sequences of death activating protein 1 (DAP-1) in the anti-sense orientation. Interestingly, DAP-1 was originally identified in a screen for positive mediators of apoptosis using anti-sense cDNA libraries to rescue cells from cell death in the presence of interferon gamma (IFN-γ) (2). Because DAP-1 exhibited the strongest phenotype of the four cDNAs (20- to 25-fold increase in invasion), and because it has been shown previously to have tumor suppressor activity (3), it was selected for further analysis. Interestingly, expression of anti-sense DAP-1 in NIH 3T3 cells is associated with transformation (as measured by focus formation), and activation of the small GTPase RhoA. A detailed description of the isolation, and characterization of DAP-1 is included below.

BODY:

Recovery of proviral inserts from a screen of the MBA-MB-231 library: In year 1 of this proposal we described the construction and initial screening of an MBA-MB-231 derived expression library using MCF-7 cells as recipients. This screen yielded 5 mass populations of cells that were enriched for invasive cells. PCR-based screening of genomic DNAs derived from each cell population identified between 2 to 5 proviral inserts (Fig.1A) per cell population. Genomic Southerns were performed to confirm the presence of the provirus using a vector-specific probe (*sup*F) (not shown). Any PCR bands that were confirmed by Southern blot were excised from the gel, recloned into the retroviral vector pCTV3H, and analyzed by restriction digest (Fig. 1B). A total of 8 clones were recovered in this manner. *Rsa*1 footprinting revealed that 3 of the clones were identical (Fig. 1C). We were unsuccessful in cloning two bands (designated 3.3 and 5.1) that hybridized with the *sup*F probe. Two additional bands (designated 3.1 and 3.3), and the ubiquitous 0.5 kb band, did not hybridize with the *sup*F probe, and were presumed to be non-specific PCR products.

Confirmation of invasive potential of proviral inserts: The 8 recovered clones (pCTV3H versions) were repackaged, and used to individually infect MCF-7 cells. Cell populations were selected with hygromycin B, and then tested for invasiveness using a transwell assay (Table 1). Cognate vector was included as a standard for comparison. Briefly, 1×10^5 cells were serum-starved, plated in triplicate into the top chamber of a collagen-coated transwell, and then allowed to invade for 24 hours through a serum gradient. The cells on the top of the transwell were then removed, and the cells on the
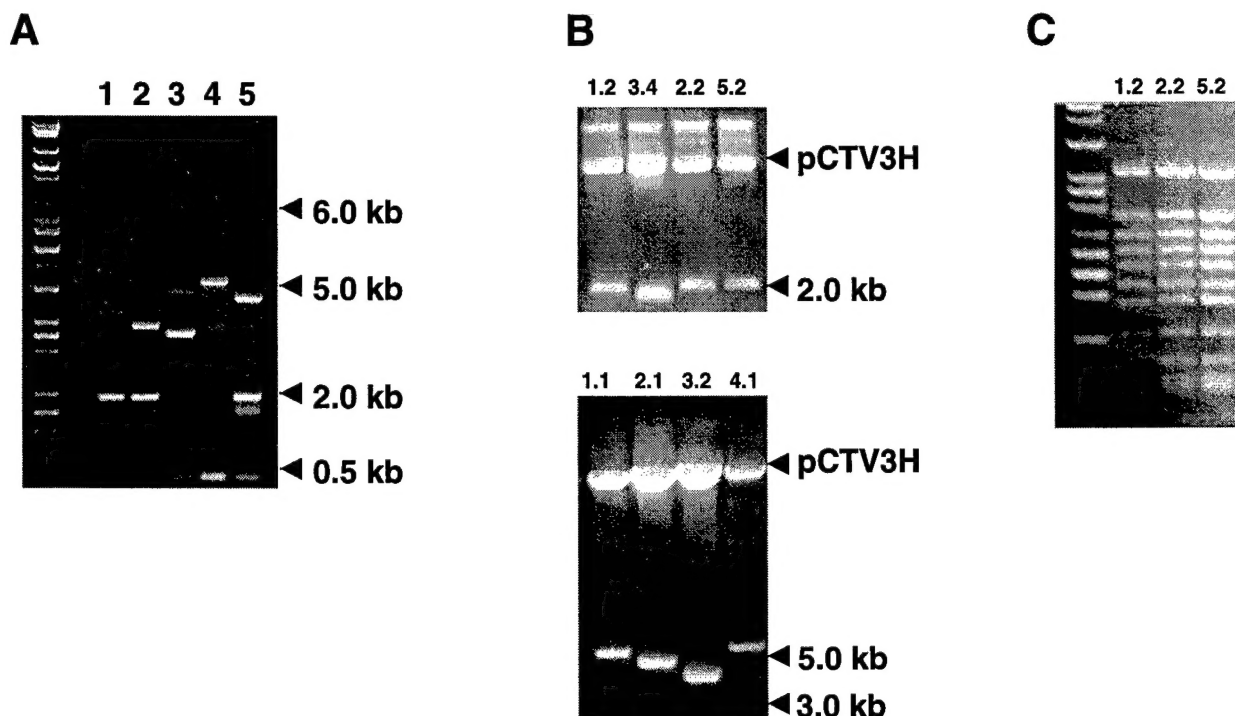
Figure 1: Recovery of proviral inserts. (A) PCR amplification of genomic DNA isolated from mass populations of cells that have been serially enriched for invasive clones. MCF-7-EcoR cells were stably infected with retroviral particles derived from the MBA-MB-231-S library. Five cell populations (50,000 cells each) were screened for invasive clones. PCR bands indicate the presence of proviral inserts within the cell populations. (B) A total of eight proviruses were recovered from the five mass populations. PCR bands were excised from the gel, digested with *Mlu*I and *Bsl*N1, and cloned into the pCTV3 retroviral vector for retesting. Restriction digests of the recovered clones revealed a cDNA size that was consistent with the PCR fragment that was targeted. (C) Because of the similarity of size of the 1.2, 2.2, and 5.2 clones, they were compared by *Rsa*I restriction digest footprinting. All three have an identical footprint suggesting that they are identical clones.

Table 1: Invasive properties and sequence identity of recovered cDNAs

Clone Designation	% invasion[a] (X10⁻⁵)	Clone Identification (genbank accession # / nucleotide residues)
pCTV3H	3 +/- 1	
1.1	10 +/- 2	Hormone Sensitive Lipase (LIPE) (NM005357 / nuc.1-3805)
1.2	67 +/- 12	DAP-1 antisense (X76105 / nuc. 2045-68)
2.1	13 +/- 4	Heat Shock 70kD Protein5 (HSPA5) (NM005347 / nuc. 5-3461)
2.2	71 +/- 8	DAP-1 antisense (X76105 / nuc. 2045-68)
3.2	2 +/- 1	Not done
3.4	4 +/-2	Not done
4.1	21 +/- 5	Actin Binding LIM protein (ABLIM) (NM006719 / nuc. 502-6365)
5.2	83 +/- 13	DAP-1 antisense (X76105 / nuc. 2045-68)

[a]MCF-7 stable cell lines were generated from recovered proviral inserts, and were then tested individually for invasive properties. One x 10⁵ cells were seeded in the upper well of a transwell invasion chamber. At 24 hr, cells that had reached the lower well were stained, counted, and expressed as a fraction of the total cells seeded.

underside were fixed, stained and counted. Six of the 8 recovered clones (including the three identical clones) exhibited increased invasiveness ranging from 2- to 20-fold.

Identity of recovered clones: The identity of the four invasive clones was determined by DNA sequencing (Table 1). Proteins that were identified included an actin binding protein (ABLIM), a lipase (LIPE), a heat shock protein (HSPA5), and DAP-1.

ABLIM is an actin binding protein that is thought to mediate interactions between actin filaments, and cytoplasmic signaling molecules (4). It consists of a COOH-terminal cytoskeletal domain, which is 50% identical to erythrocyte Dematin, and an NH_2-terminal domain consisting of four double zinc fingers, which conform to the LIM motif consensus sequence (4). ABLIM binds to F-actin through its Dematin like domain *in vitro*, and may form a bridge to an array of potential LIM protein-binding partners. Interestingly, ABLIM maps to a region of chromosome 10q25 that is frequently the site of loss of heterozygosity in human tumors (5). The clone that we identified is a truncated version of this protein that is missing some of the NH_2-terminal zinc finger motifs, as well as COOH-terminal sequences.

LIPE is an 84-kDa cholesterol esterase and triglyceride hydrolase that functions in the release of fatty acids from adipocytes (6). This hormone sensitive lipase plays an important role in adipose tissue metabolism, obesity and diabetes, as well is in mouse fertility.

HSPA5 is also known as glucose regulatory protein 78 / immunoglobulin heavy chain binding protein (Grp78/BiP), and is a molecular chaperone that is involved in polypeptide translocation across the ER membrane (7). HSPA5 expression is induced by physiological stress such as glucose deprivation and hypoxia, and is thought to protect the cell from stress-induced cell death. It does this by forming a complex with caspase 12 in the ER, thus preventing its release (8). This cytoprotective effect of HSPA5 may contribute to the malignant progression and resistance to radiation and chemotherapy seen in solid tumors that are in hypoxic and starved conditions.

DAP-1 was independently isolated three times, and is the only clone that is orientated in the anti-sense. In addition, it was by far the most invasive of the recovered clones (approximately 20- to 25-fold over vector). DAP-1 (an acronym for Death Associated Protein 1) is expressed as a single 2.4 kb mRNA that encodes for a basic, proline-rich, cytoplasmic 15 kDa protein (2). DAP-1 was originally identified as an anti sense clone in a screen for positive mediators of IFN-γ-mediated apoptosis (2). Extensive studies have now shown that DAP-1 is phosphorylated by a calcium/calmodulin-dependent DAP kinase which was recently found to have strong tumor suppressive activities, thus coupling the control of apoptosis to metastasis (3). DAP kinase is localized to actin filaments and this localization is critical for the apoptotic process, specifically to the disruption of stress fibers, a hallmark of apoptosis. Both these genes appear not only to be involved in cell death in response to IFN-γ, but also to apoptosis as a result of detachment from the extracellular matrix. In our screen we have isolated a 1,977 bp cDNA (nucleotide residues 68-2045) of DAP-1 in the anti-sense orientation. Therefore, it is presumably through downregulation of native DAP-1

that this clone causes an increase in invasion.   Because of the potency of the invasiveness seen with the DAP-1 clone, it was selected for a more detailed analysis.
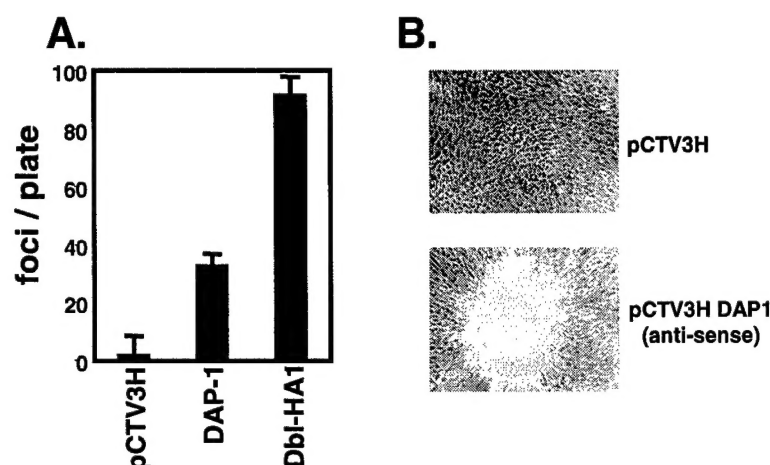
A.

B.

pCTV3H

pCTV3H DAP1
(anti-sense)

Figure 2: Transforming activity of DAP-1 (anti-sense) in NIH 3T3 mouse fibroblasts. (A) Cells were transfected by calcium phosphate coprecipitation with 3 µg (per 6 cm dish) of the indicated cDNAs, and foci were stained and counted at 14 days. (B) Representative focus induced by expression of anti-sense DAP-1 in NIH 3T3 cells.

Expression of DAP-1 in the anti-sense causes focus formation in NIH 3T3 cells. Because downregulation of DAP-1 is associated with increased invasion, and because it has been implicated in the regulation of the actin cytoskeleton, we were interested to see if DAP-1 would have transforming activity in NIH 3T3 cells. Towards this end, we carried out an NIH 3T3 primary focus assay, comparing the transforming activity of anti-sense DAP-1 to the RhoGEF Dbl. We have shown previously that Dbl has potent transforming activity in this cell type (9), and it was included as a positive control. Cognate vector was also included in this analysis as a negative control. Similar to Dbl, expression of anti-sense DAP-1 in NIH 3T3 cells was associated with focus-forming activity (Fig. 2A). The morphology of the foci were highly reminiscent of those induced by activated derivatives of the Rho family GTPases (Fig. 2B).

Expression of DAP-1 in the anti-sense is associated with activation of RhoA (but not Rac1 or Cdc42) in NIH 3T3 cells. Since DAP-1 produced a phenotype in NIH 3T3 cells that we have observed previously with activated Rho proteins, we wondered whether downregulation of DAP-1 in these cells was associated with increased GTP-loading on Rho.   To test this hypothesis, a stable cell line was generated with the DAP-1 anti-sense clone. Lysates were collected, and then subjected to affinity purification to measure levels of endogenous RhoA, Rac1 and Cdc42 (Fig. 3). We routinely use these assays to measure the in vivo activity of Rho family guanine nucleotide exchange factors (9).   Compared to the vector cell lines, we were consistently able to detect elevated levels of RhoA (Fig. 3A), but not Rac1 (Fig. 3B) or Cdc42 (Fig. 3C) in our stable cell line. Thus, downregulation of DAP-1 in NIH 3T3 cells is associated with increased RhoA loading, which probably accounts for its transforming activity.
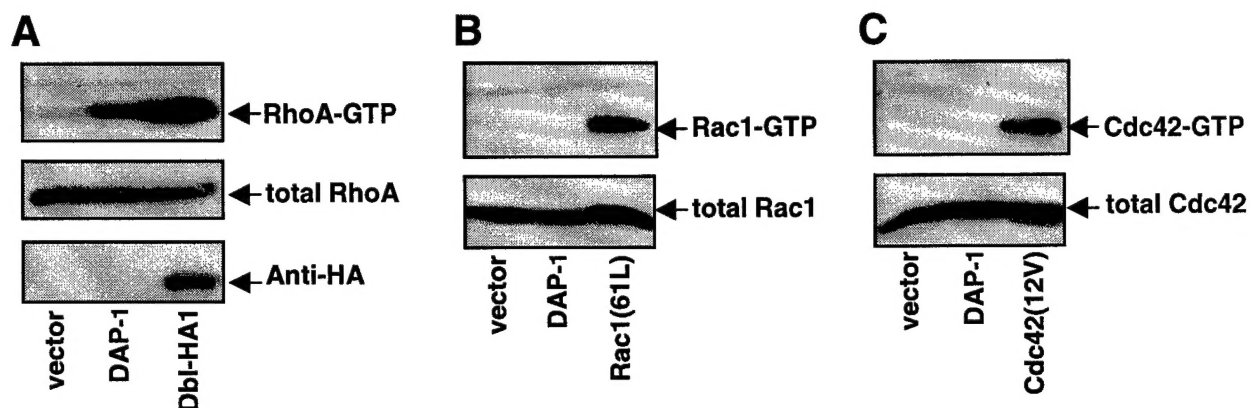
Figure 3: Anti-sense DAP-1 activates RhoA when stably expressed in NIH 3T3 cells. Lysates were collected from NIH 3T3 cells that stably express the indicated plasmids. Lysates were split into three parts, each of which was normalized for expression of either RhoA (A), Rac1(B) or Cdc42 (C). Each lysate was then subjected to affinity purification using immobilized GST-Pak (Rac1 and Cdc42) or GST-C21 (RhoA). GTP-bound Cdc42 (*B*: GTP-Cdc42) and Rac1 (*C*: GTP-Rac1) that were precipitated with GST-PAK, or GTP-bound RhoA (*A*: GTP-RhoA) that was precipitated with GST-C21, were visualized by Western blot. Dbl-HA1, Rac(61L), and Cdc42(12V) were included as positive controls for RhoA, Rac1 and Cdc42 respectively.

KEY RESEARCH ACCOMPLISHMENTS:

➢ Recovery of 8 proviral inserts from 5 sequentially enriched populations of MCF-7 cells that were stably infected with retroviral particles derived from the MBA-MB-231S library.

➢ Generation of MCF-7 stable cell lines from the 8 recovered proviral inserts and confirmation of invasive potential in 6 of the 8 clones.

➢ Complete sequencing of four independent, invasion-inducing cDNAs. One clone identified as the tumor suppressor DAP-1, expressed in the anti-sense.

➢ Analysis of the transforming and signaling potential of DAP-1 in NIH 3T3 cells reveals that it has the properties of a general tumor suppressor that may function by deregulating the small GTPase RhoA.

REPORTABLE OUTCOMES:

None

CONCLUSIONS:

The work performed during this year establishes that our retrovirus-based system can be used to identify cDNAs that result in the increased invasive potential of MCF-7 cells. As expected, a considerable amount of time was spent recovering and retesting proviral inserts. Importantly, an extremely low frequency of false positives was observed which bodes well for future screens. Now that we have established both our screening and recovery conditions, we have initiated a larger scale screen using additional libraries that we generated during Year 1

A very potent inducer of invasion (anti-sense DAP-1), was isolated in 3 independent mass populations of motile cells, and was selected for further analysis. We will continue with the analysis of this cDNA in the upcoming year. In particular, we will be examining the status of Rho proteins in MCF-7 cells that stably express DAP-1 (anti-sense) to see if we can recapitulate our results from the NIH 3T3 cells. Additionally, we will also begin our analysis of the other cDNAs that were recovered in our screen.

To summarize, we have now completed Tasks 1 and 2 in the original Summary of Work, and are currently progressing in Task 3. However, we will not limit our efforts to the analysis of the cDNAs in hand, but will continue to screen for new cDNAs.

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